1. Introduction

Chemotherapy, in specific clinical settings, is an effective modality of cancer treatment. For example, progress has been made in the treatment of Hodgkin's disease, large-cell lymphoma, acute lymphocytic leukemia, and testicular cancer to the point where these entities are now considered to be "curable." In addition, with the development of combination chemotherapy, other malignancies, such as ovarian cancer, small-cell lung cancer, and advanced breast cancer, although not yet curable, demonstrate meaningful clinical responses to therapy in many patients. Unfortunately, when a relapse occurs, it is often associated with the development of drug-resistant tumors, thus limiting additional response to chemotherapy.

Failure of a chemotherapeutic regimen to induce a response may be due to many factors. Foremost among these is the inability of the drugs to reach the critical cellular target. In this regard physiological factors may play a large role in the successful outcome of therapy. Absorption, distribution, metabolism, and elimination are key principles in successful cancer chemotherapy, and while they should not be deemphasized as possible reasons for a cell's lack of response to therapy, the focus of this chapter is on the alterations that occur at the cellular level, specifically those associated with the development of multidrug resistance.

A significant proportion of patients with large tumor burdens at the time of initial diagnosis eventually undergo a relapse of their disease and die as a consequence of drug-resistant malignancies. The finding that increased tumor burden is associated with reduced response rates is compatible with the Goldie–Coldman (G11) model showing that spontaneous mutations occur which confer drug resistance, and that mutation-derived clones will eventually dominate as drug-resistant cells are selected by continued treatment. For tumor types with an overall poor response rate to chemotherapy, the tumors are considered to have intrinsic drug resistance. Such intrinsic resistance appears to be the rule for colon cancer, adenocarcinoma of the lung, and disseminated malignant melanoma. This form of drug resistance is in contrast to the situation observed in leukemias and lymphomas, in which patients initially respond to chemotherapy but often relapse and become refractory to further treatment with the same drugs. Patients who convert from a drug-sensitive to a drug-resistant state are considered to have acquired drug resistance.

It is necessary to understand the underlying molecular mechanisms responsible for the development of drug resistance in order to devise rational therapeutic approaches aimed at reversing or preventing the emergence of refractory tumors. A number of different mechanisms have been shown to account for various types of drug resistance. Examples of such include the resistance to methotrexate resulting from increased expression of dihydrofolate reductase, resistance to BCNU (carmustine) due to overexpression of O^6-methylguanine DNA methyltransferase, resistance to nitrogen mustard compounds as a consequence of elevated glutathione and/or glutathione transferase activity, and resistance to cisplatin as a result of elevated metallothionein levels. One of the most thoroughly studied drug resistance mechanisms is mediated by an isoform of P-glycoprotein and results in a broad spectrum of resistance to chemotherapeutic agents.

2. Multidrug Resistance

2.1. Historical Significance

Multidrug resistance (MDR) represents a major challenge in cancer chemotherapy, since it limits the effectiveness of many chemotherapeutic agents. The phenomenon of MDR was first described in the 1960s by Kessel *et al.* (K10), who noted that P388 murine leukemia cells, selected for resistance to vinblastine, became cross-resistant to actinomycin D, daunorubicin, and the vinca alkaloids. These cells displayed a decrease in drug accumulation which paralleled their degree of resistance. In 1970 Biedler and Riehm (B16) reported that Chinese hamster lung cells which had been selected *in vitro* for resistance to actinomycin D developed cross-resistance to the vinca alkaloids, daunomycin, and mitomycin C. Ling and colleagues (B4) made similar observations in Chinese hamster ovary (CHO) cells, in which they observed that mutant CHO cells selected for resistance to colchicine also developed cross-resistance to a number of unrelated compounds, including daunomycin and vinblastine. In addition to their cross-resistance profile, these cells also displayed a decrease in drug accumulation which correlated with their level of resistance (L5) and overexpressed an integral membrane glycoprotein of approximately 170 kDa, termed "P-glycoprotein," which was not found in the drug-sensitive parental CHO cells (J2). The degree of expression of this glycoprotein was shown to generally correlate with the level of drug resistance and decreased drug accumulation observed in these cells (K6, L3). This suggested a functional role for the P-glycoprotein in terms of modulating the levels of drug accumulation in the resistant cells. Thus, it was found that the development of resistance to a single cytotoxic drug in the laboratory setting could lead to the simultaneous development of cross-resistance to a number of other, unrelated, compounds.

2.2. MDR Phenotype

Since these initial reports many investigators have described multidrug-resistant tumor cell lines selected *in vitro* with a number of natural-product antineoplastic agents (B8, B10, N3). A common feature of these drug-resistant lines is the expression of the P-glycoprotein (G8). Cell lines that display the

MDR phenotype are resistant to the vinca alkaloids, anthracyclines, epipodophyllotoxins, taxol, and actinomycin D (see Table 1). They generally retain sensitivity to alkylating agents and antimetabolites. Resistance in these cells is generally secondary to an increased energy-dependent drug efflux, resulting in a decreased intracellular drug accumulation (E2). Most of the drugs involved in MDR cross-resistance are amphipathic and readily diffuse across cell membranes without the aid of specific transport systems. Although, in general, they have complex ring structures and a positively charged nitrogen atom, many of these drugs do not share a common cellular target, and it was this fact that focused the attention of early investigators on the central role of the plasma membrane in the MDR phenomenon. In humans two closely related genes, *MDR1* and *MDR2* (the so-called "multidrug resistance" genes), encode highly homologous P-glycoproteins. Only the *MDR1* gene has been linked to the MDR phenomenon, however. The product of the *MDR1* gene, P-glycoprotein, is a pleiotropic 170-kDa membrane transport protein characterized by its ability to carry out energy-driven transport of a wide variety of structurally and functionally unrelated compounds. Consistent with its function as a transport pump, the expression of P-glycoprotein in normal tissues suggests a physiological role for the transport of steroids, bilirubin, carcinogens, and other xenobiotics (see Section 7.1).

3. *MDR1* Gene Family

3.1. Molecular Analysis of *MDR* Genes

Consistent with the Goldie-Coldman (G11) hypothesis that genetic alterations lie at the heart of the development of a resistant cell population, a number of groups have identified amplified DNA sequences from multidrug-resistant cell lines (F10, G24, M15, S9, V4). These amplifications were present as either

TABLE 1
Chemotherapeutic Drugs Involved in Multidrug Resistance

Actinomycin D
Daunorubicin
Doxorubicin
Etoposide (VP-16)
Mitoxantrone
Taxol
Teniposide (VM-26)
Vinblastine
Vincristine

homogeneously staining regions or double-minute chromosomes. Fojo *et al.* (F10) demonstrated that four different drug-resistant human carcinoma cell lines shared common amplified regions which their drug-sensitive counterparts lacked. These amplified sequences were shown to contain a small family of at least five related genes (V4). One of these genes, the *MDR1* gene, was found to encode a 4.5 to 5.0-kb mRNA which in turn coded for P-glycoprotein (V4).

DNA transfection studies have demonstrated a causative role for the *MDR1* gene. Genomic DNA isolated from multidrug-resistant cells was found to be capable of conferring the MDR phenotype, including the expression of P-glycoprotein, when transfected into drug-sensitive cell lines (D9, D11, S13). This methodology does not preclude the cotransfection of other genes during the experimental process, however, and it is possible that other genes were responsible for the observed findings. To overcome this limitation, Gros *et al.* (G22) transfected a full-length cDNA of the *MDR1* gene into drug-sensitive mouse cells and found that the transfectants had acquired the MDR phenotype. Ueda *et al.* (U2), using a cDNA coding for the human *MDR1*, obtained similar results. Others have used a retroviral gene transfer methodology to transfer full-length *MDR1* cDNA into drug-sensitive cells and have obtained identical results (G28, P2). Thus, full-length functional cDNAs for *MDR* genes have been shown to confer the full MDR phenotype on drug-sensitive cells.

Not all multidrug-resistant cell lines contain amplified *MDR1* regions, however (A2, D2, D17). Shen *et al.* (S13) demonstrated that an increase in the expression of the 4.5-kb *MDR1* mRNA can precede gene amplification in multidrug-resistant human leukemia and ovarian carcinoma cells. Sugimoto *et al.* (S26) have also demonstrated that in revertant cells of a human multidrug-resistant leukemia line there is a decrease in the expression of the *MDR1* gene without loss of its amplification. Using a retroviral expression vector containing a full-length *MDR* cDNA, Guild *et al.* (G28) have demonstrated that transfer of a single copy of the *MDR* cDNA to initially drug-sensitive fibroblasts was sufficient to confer a high-level MDR phenotype without prior drug selection. These findings leave open the question of the relevance of gene amplification in the clinical situation, in which low levels of drug resistance are more likely to be encountered (F17, I6, M13). In this setting all that may be needed for development of the MDR phenotype is slight overexpression of the *MDR1* message without the requirement for gene amplification.

The human *MDR1* gene was localized to chromosome 7, band q21.1 (C1, C15). The protein-coding portion of this gene contains 27 exons, 14 coding for the left and 13 coding for the right half of the protein (R13). There does not appear to be any correlation between individual exons and specific structural elements of the protein.

Genetic analysis has also revealed the existence of more than one *MDR* gene in mice, hamsters, and humans (D12, D15, G26, G27, J3, R5). Studies by Ling's

group in Toronto (J3) have demonstrated that the hamster P-glycoprotein gene family is composed of three members: *pgp1*, *pgp2*, and *pgp3*. The mouse *MDR* gene family has been characterized and also found to have three members, designated *mdr1a*, *mdr1b*, and *mdr2* (G27). The mouse genes corresponding to the human *MDR1* gene are *mdr1a* (also referred to as *mdr1*) and *mdr2a* (also known as *mdr3*), and both encode functional transporters. The murine *mdr2* gene does not appear to be involved in drug resistance. In humans there is evidence for a second human *MDR* gene, designated the *MDR3* gene (also known as *MDR2*) (J3, M11, V2). The two members of the human *MDR* gene family encode 4.1- to 4.5-kb species of RNA that have a high degree of homology (J3, M11, V2). The functional significance of this finding in the development of drug resistance is unclear at the present. It has not been shown that the product of the *MDR2* gene, the Mdr2 protein, functions as a transporter. Nevertheless, it is referred to as an MDR protein because of its structural relationship to P-glycoprotein (K4). Members of the *MDR* gene family from different species can be categorized into classes based on sequence similarity of the 3′-untranslated regions (N1). Relationships and the current nomenclature of the *MDR* genes in several species are summarized in Table 2.

3.2. *MDR1* Regulation

While increased *MDR1* mRNA has been observed as a consequence of gene amplification in both rodent and human cell lines, increases in mRNA expression have also been observed in the absence of amplification. Such findings suggest that the *MDR1* gene may be transcriptionally and/or translationally regulated (B21, S12).

The *MDR1* gene has at least two promoter regions (R16, U3, U6). Using primer extension analysis and S1 nuclease mapping, Ueda *et al.* (U3, U6) ascertained that transcription of the human *MDR1* gene may be initiated from a major downstream promoter in which transcription may begin at positions 136–140 or

TABLE 2
NOMENCLATURE FOR MULTIDRUG-RESISTANCE GENES

	Designation		
Species	Class I	Class II	Class III
Human	*MDR1*	—	*MDR3*[a]
Mouse	*mdr3*	*mdr1*	*mdr2*
Hamster	*pgp1*	*pgp2*	*pgp3*

[a]Also known as *MDR2*. (Adapted from Ref. J3.)

155–180 and from a minor upstream promoter which was found to be expressed in colchicine-, but not doxorubicin- or vinblastine-selected KB cells. Most normal tissues have been found to utilize the downstream *MDR1* promoter (C15, U6).

The major (downstream) promoter of human *MDR1* has a CAAT box and two GC-rich regions (putative SP-1 binding sites), but lacks a TATA box (U3). This lack of a TATA box has been associated with heterogeneity of transcription sites in eukaryotic cells (M3). *In vitro* studies using HeLa cell nuclear extracts and *MDR–CAT* (chloramphenicol acetyltransferase) fusion vectors have identified two downstream sequences that influence expression of the human *MDR1* gene and are essential for proper initiation of transcription (C28, M2).

Several potential transcriptional regulatory elements have been found within the human *MDR1* gene, including several heat shock consensus elements and a phorbol ester response element (A4, C18). Studies have also demonstrated that heat shock, cadmium chloride, and sodium arsenite may induce *MDR1* mRNA (C16, C18). This induction was found to be sensitive to actinomycin D, indicating a requirement for new RNA (C18). *MDR–CAT* reporter gene constructs have demonstrated that mutant *Ras* and *p53* genes may stimulate the *MDR1* promoter (C19). Recent data also suggest that the *MDR1* gene is transcriptionally regulated through a signal transduction pathway involving the c-Raf kinase (C31).

Treatment of human colon cancer cell lines with differentiating agents such as dimethyl sulfoxide or sodium butyrate has been shown to increase *MDR1* gene expression (M19). Treatment with cytotoxic agents, ultraviolet radiation, or partial hepatectomy has also been shown to increase *MDR1* expression in both rodent and human cell lines (B24, C12, C17, M6, M19, T6, U1). The increase observed following exposure to antineoplastic agents, including ones not transported by P-glycoprotein, persisted for several weeks following removal of the drug (C12). The drug-mediated *MDR1* induction was blocked by nonspecific protein kinase inhibitors.

The DNA sequences of mouse and hamster *mdr* promoters differs greatly from that of the human, which perhaps explains why the rodent promoters are more responsive to certain kinds of environmental stresses. The murine promoters contain both TATA and CAAT boxes as well as putative SP-1, AP-1, and AP-2 sites (C21, H18, I1, R5). There appears to be more than one *mdr1a* promoter, which, together with alternative polyadenylation sites, may account for the multiplicity of *mdr1a* transcripts observed in mouse cells (H18).

Expression of *MDR1* has been observed to be elevated in the gravid mouse uterus, suggesting that its expression may be under hormonal influence (A5, A6). The *mdr1b* promoter, which drives expression of P-glycoprotein in the adrenal and secretory glands of the endometrium, has been shown to contain a progesterone response element (C21).

4. Characterization of the P-Glycoprotein

4.1. Structure and Function

The P-glycoprotein, as mentioned in the previous section, is coded for by the *MDR1* gene and is a 1280-amino-acid protein expressed as a single polypeptide chain containing two homologous portions of equal length (C14, G7, G23, U4). The hydropathy profile of its amino acid sequence indicates 12 hydrophobic regions, consistent with transmembrane loops *in situ*. The overall structure is suggestive of that of a transmembrane channel. There are two cytoplasmic nucleotide binding regions located near the C-terminal portion of the P-glycoprotein on the inside of the membrane which are involved in ATP binding and hydrolysis (A3). The polypeptide component of the P-glycoprotein has a molecular weight of 120–140 kDa. The predicted amino acid sequence and structure of P-glycoprotein are similar to those of other membrane-associated transport proteins, such as the bacterial transport protein hemolysin B (G7). Findings such as this have further supported the role of P-glycoprotein as an energy-dependent drug efflux pump.

There is also evidence suggesting that the P-glycoprotein may exist as dimers or tetramers *in situ* (B20).

Using the monoclonal antibody MRK16, Willingham *et al.* (W9) localized the P-glycoprotein by immunofluorescence and electron microscopy to the external surface of the plasma membrane and the luminal side of Golgi stack membranes in multidrug-resistant human carcinoma cells. It was not found in coated pits on the plasma membrane and was absent from lysosomes. P-glycoprotein has also been detected in normal tissues, which suggests a functional role outside of antitumor agent resistance.

The Mdr2 protein isoform shares a high level of sequence homology with the P-glycoprotein in humans and mice, with >75% identical amino acid residues (C15, V3). The more conserved regions are those including or adjacent to the nucleotide binding sites within the cytoplasmic domains of the P-glycoproteins. Therefore, nucleic acid probes constructed to distinguish *MDR1* and *MDR2* RNAs are targeted to highly divergent sequences corresponding to the more dissimilar amino acid sequences at the exterior surface of the membrane.

4.2. Drug Transport and Binding

As alluded to earlier, one of the most common alterations observed in MDR tumor cells is a decrease in drug accumulation (B6, D2, D8, F6, I3, K10, L3, R6, S16). Such changes may be brought about by a reduction in drug influx, an alteration in intracellular drug binding, or an enhancement of drug efflux. Most

of the drugs involved in MDR cross-resistance are amphipathic and readily cross cell membranes without the aid of specific transport systems.

Studies by Dano (D8) and others (I2, I4, S16) have demonstrated the possibility that an active energy-requiring drug efflux "pump" in the resistant cells may be responsible for the decreased drug levels. When resistant cells were depleted of ATP, either by removing glucose from the culture medium or by adding metabolic poisons such as sodium azide, cyanide, or iodacetate, there was an increase in the steady-state drug concentrations as compared to the drug-sensitive counterparts. When these energy-deprived drug-resistant cells were then placed back into a growth medium containing glucose, drug levels again declined, lending support to the idea of an energy-dependent drug efflux pump.

Using radiolabeled photoaffinity derivatives of vinblastine, investigators showed that this compound bound to an integral membrane glycoprotein of the same molecular weight as P-glycoprotein. Furthermore, this drug–protein complex could be precipitated from solution by the addition of a polyclonal antibody to P-glycoprotein (C29, C30, S1). Such binding was shown to be specific and was inhibited by addition of cold vinblastine, verapamil, or daunorubicin. These results suggest that P-glycoprotein serves as a possible drug-binding protein in the resistant cells. Other photoaffinity analogs, such as [^{3}H]azidopine and [^{125}I]iodoarylprazosin, also bind to a membrane glycoprotein of the same size as P-glycoprotein (G18, S2, Y3). In the case of [^{3}H]azidopine, the specificity of binding was established by competitive blocking with cold azidopine, nitrendipine, and nifedipine as well as by verapamil and diltiazem. The photolabeling due to [^{3}H]azidopine was also inhibited by addition of doxorubicin, vinblastine, and actinomycin D.

Using plasma membrane vesicles prepared from multidrug-resistant cells in an inside-out manner, it has been possible to demonstrate [^{3}H]vinblastine transport (H15, H16, L2). Such transport occurred against a concentration gradient, required a constant supply of energy, and did not occur when vesicles were prepared from drug-sensitive cells lacking the P-glycoprotein. Nonhydrolyzable ATP analogs such as AMPPNP did not support this transport (H15, L2). Others have demonstrated similar transport of chemotherapeutic drugs in vesicles made from P-glycoprotein-expressing cells, further supporting the transport role of this molecule (K2, T3).

The prevailing hypothesis is that P-glycoprotein is functioning as a transmembrane pore-forming protein leading to an energy-dependent outward drug efflux. The identification of ATPase activity associated with P-glycoprotein was significant because this provided a mechanism by which energy may be transduced for active drug efflux, but the exact nature of the drug efflux mechanism is not fully understood. Initially, it was believed that drugs were removed from the cytoplasm through a single pore formed by P-glycoprotein. Others have proposed models in which P-glycoprotein acts as a "flippase," flipping the drug from the

inner leaflet of the lipid bilayer to the outer leaflet (H8), or as a "hydrophobic vacuum cleaner," in which drugs are removed while in transit through the plasma membrane before they reach the cytoplasm (G16).

4.3. Mutational Analysis

One of the major questions yet to be answered is how one molecule can recognize and transport such a wide variety of substrates. While not answering this question, the analysis of mutant P-glycoproteins has shed some light on the functioning of this molecule.

Choi *et al.* (C20) were the first to report a mutant P-glycoprotein. Using a human carcinoma cell line, they described a Gly-to-Val substitution at amino acid 185 which was shown to alter the cross-resistance profile of a human carcinoma cell line expressing this molecule. The mutation resulted in an enhancement of colchicine transport, while diminishing that of vinblastine and actinomycin D.

Devine *et al.* (D13) also reported mutations in the hamster P-glycoprotein which altered the MDR phenotype. They described two substitutions, Gly to Ala at position 338 and Ala to Pro at position 339, both located in the transmembrane 6 domain, which resulted in a decreased resistance to several drugs while maintaining maximal resistance to actinomycin D.

In the mouse *mdr1* gene an altered phenotype was reported when Ser was changed to Phe at position 939 in the transmembrane 11 region (G25). This change decreased doxorubicin and colchicine transport, while leaving vinblastine transport unaffected. At position 183 in the first cytoplasmic loop, an Asn-to-Ser substitution increased resistance to vinblastine and actinomycin D (C34).

Deletions in either the C- or N-terminal portion of the molecule resulted in a loss of transport, indicating that these regions were critical to the proper functioning of the molecule (C35). Mutations in either of the two ATP binding sites have also been shown to abolish transport activity, suggesting that these two sites are not acting independently of one another (A11). Not all mutations have been shown to alter the MDR phenotype, however (C20, K12).

4.4. Posttranslational Modifications

4.4.1. *Glycosylation*

There is a single glycosylation locus in the extracellular region of the N-terminal half of the P-glycoprotein molecule. Variability in glycosylation can give rise to P-glycoproteins ranging from 135 to 180 kDa (E2, G17). Absence of the glycosylated portion of the Mdr1 protein as a result of tunicamycin or pronase treatment had no effect on the level of drug resistance in cell lines (B6), and glycosylation-defective mutants have been isolated which retain the MDR phenotype (L4), suggesting that the carbohydrate moiety is not required for P-glycoprotein activity.

4.4.2. *Phosphorylation*

Studies such as those by Carlsen *et al.* (C3) and Garman *et al.* (G4) demonstrated that the P-glycoprotein molecule was phosphorylated and dephosphorylated in the plasma membrane. It is phosphorylated on several sites, including Ser and Thr residues (H3). While not fully understood, phosphorylation of P-glycoprotein appears to modulate its activity and thus the level of drug resistance. P-glycoprotein appears to be phosphorylated in the basal state and can be further phosphorylated by treatment with phorbol esters (F3, H3). Treatment with phorbol esters resulted in a decreased vinblastine accumulation and a concomitant increase in the level of resistance in human KB–V1 cells (B3). Hamada *et al.* (H3) reported that treatment of MDR cells with chemosensitizing agents such as verapamil or trifluoperazine resulted in an increase in the level of phosphorylation of the molecule.

The exact pathway by which the P-glycoprotein is phosphorylated is not fully known, but it appears to involve several kinases, including protein kinase C, the cAMP-dependent protein kinase A, and a novel kinase which has not been fully characterized (B3, F3, M12, S24). Membrane-associated protein phosphatases 1 and 2A have been implicated in the dephosphorylation of P-glycoprotein (C6).

4.5. Other Activities

In addition to its role as a transport molecule, evidence has been put forth suggesting that P-glycoprotein may also act to regulate cell volume in selected tissues. Using NIH 3T3 cells transfected with the human *MDR1* gene, Valverde *et al.* (V1) described a volume-regulated chloride channel activity associated with these cells. The chloride channel activity of these cells was ATP dependent and was inhibited by the addition of forskolin, 1,9-dideoxyforskolin, verapamil, or quinine. Antisense oligonucleotide to the *MDR1* gene also abolished the chloride channel activity of these cells. The channels were distinct from the cAMP-regulated channels associated with the cystic fibrosis gene product, *CFTR*.

The transport and channel activities of P-glycoprotein were shown to be independent of each other through the use of directed mutagenesis studies in which the nucleotide binding site was altered (G9). The investigators of this study suggested a model in which the P-glycoprotein exists in two configurations. In one it serves a transport function, while in the other it acts as a chloride channel. The two conformations would be interchanged by alterations in cellular tonicity. The effects of transportable substrates on channel activity suggested that the transport and channel activities of P-glycoprotein were mutually exclusive (G9).

Both P-glycoprotein and *CFTR* share many structural similarities. Trezise *et al.* (T8) reported that these two proteins were expressed in a complementary fashion in rodent tissues. In addition, they described a switch from one gene product to another in certain tissues. Expression switched from *CFTR* to *MDR1* in the intestine at the crypt–villus boundary and in the uterine epithelium upon pregnancy. The significance of these findings is unclear at present.

5. ABC Superfamily of Transporters

It is now established that mammalian *MDR* genes are related to an evolutionarily conserved family of *MDR*-like genes in nonmammalian species (H9, J3). For example, P-glycoprotein shares an amino acid sequence homology with several bacterial transport systems, such as the α-hemolysin and malK transport systems in *Escherichia coli* and the hisP system in *Staphylococcus typhimurium* (C14, G7, G23). Mdr1 is a member of the ABC (ATP-binding cassette) superfamily of transporters, which includes, in addition to bacterial transporters and P-glycoprotein, a pigment transporter in *Drosophila melanogaster* (O1), a pump that appears to mediate chloroquine resistance in *Plasmodium falciparum* (*pfmdr*) (F11, W10), a transporter for the α-peptide making factor of yeast (*STE6*) (K16, M8), two linked genes associated with transport of peptides into the endoplasmic reticulum for class I antigen presentation (*TaP1* and *TaP2*) (M22), as well as *CFTR* and a novel gene associated with multidrug resistance in a human lung cancer cell line (C22, J3) (Table 3). To date there are over 40 members of the ABC family, most of them transporters (H9, J3). They share structural similarities, which include a set of six hydrophobic transmembrane

TABLE 3
MEMBERS OF THE ATP-BINDING CASSETTE (ABC) SUPERFAMILY OF TRANSPORTERS

Bacterial nutrient transporters
malK (maltose)
hisP (histidine)
Peptide/drug transport
hylB (hemolysin)
OppD and OppF (oligopeptides)
Mdr1
pfmdr (*Plasmodium falciparum*)
STE6 (yeast sex peptide α)
Tap1 and Tap2 (antigen presentation)
Unknown function
CFTR (cystic fibrosis)
MRP

domains and a nucleotide binding region often referred to as the Walker motif (W1). In eukaryotic systems the transmembrane domains may be associated with a single ATP binding site, as observed in the Tap1 and Tap2 proteins, or they may be fused to form 12 transmembrane regions and two ATP binding sites, as found in the Mdr1 and CFTR proteins.

6. *MDR1* Gene and Protein Measurements

There is a general consensus that measurement of P-glycoprotein or *MDR1* expression in tumor samples is likely to be beneficial. Determining the sensitivity and resistance of an organism before treatment has been the standard of care in infectious disease for many years, while in oncology treatment has traditionally been initiated according to the tumor histology. Attempts to individualize cancer chemotherapy date back to the 1950s with the studies by Black and Speer (B17). A number of *in vitro* chemosensitivity assays have since been utilized for assessment of a given tumor's response to chemotherapy. All of these assays have as their basic assumption that a quantitative relationship exists between the dose of the drug and the response of the tumor cell to its cytotoxic actions. When Salmon *et al.* (S5) reported the results of their human tumor stem cell assay in the late 1970s, the routine use of *in vitro* chemosensitivity testing to individualize therapy in the clinical setting was considered almost a reality for cancer patients. Their paper described a quantitative *in vitro* assay which was suitable for the direct bioassay of tumor stem cells from patient biopsy samples. Unique patterns of sensitivity and resistance to a number of drugs were observed for individual patients and there was a highly significant correlation ($p < 0.00001$) between the *in vitro* data and the clinical response in the patient. Yet more than a decade later, in spite of refinements in cell culture conditions and automation of many of the assays, we are not much closer to this goal. In contrast to the clinical situation, the use of *in vitro* chemosensitivity testing on human tumor cell lines has become widespread in the laboratory for screening potential new agents, for understanding mechanisms of drug action, and for better understanding drug resistance.

The advantages of reliable chemosensitivity testing are numerous. Most obviously, patients whose tumors are likely to respond to chemotherapeutic drugs may be readily identified. Ineffective drugs in a particular regimen may also be identified and eliminated, thereby allowing the oncologist to escalate the dose of the effective drugs in order to tailor the chemotherapy to an individual patient's need. By identifying inactive drugs, the patient will be spared being subjected to "standard" chemotherapy regimens and their associated toxicities. In cases in which the tumor is unresponsive to chemotherapy, the oncologist may be able to

offer alternative or experimental therapies much sooner, when they might have a better chance of succeeding.

If a particular assay system is to be considered useful in predicting an appropriate therapeutic regimen, several questions must be addressed: (1) Are results obtained *in vitro* predictive of those achieved *in vivo?* (2) Are the patterns of drug sensitivities observed *in vitro* with tumors of a specific histological type similar to that observed clinically for the same tumor type? And importantly, (3) can chemotherapy chosen on the basis of *in vitro* studies improve patient survival?

When an *in vitro* chemosensitivity assay does not correctly predict sensitivity to a given antineoplastic agent in a primary human tumor biopsy, the physician must determine why the assay failed. Reasons for an assay failing to correctly predict chemosensitivity would include (1) the administration of an inadequate dose of the drug or poor drug absorption, (2) inadequate delivery of the drug to the tumor due to poor vascularization or to the presence of pharmacological sanctuaries such as the blood–brain barrier or the blood–testis barrier, (3) increased enzymatic activity leading to drug detoxification, (4) decreased activity of enzymes which activate drugs, (5) alterations in the growth rate of the tumor from the *in vivo* to the *in vitro* state, and (6) the problem of tumor heterogeneity. This last point is one of the largest concerns with *in vitro* testing. It cannot be established with certainty that the biopsy material used in these assays is truly representative of the patient's entire tumor.

6.1. Cell Culture Assays versus Molecular Probe Assays

The last several years have witnessed tremendous progress in the ability to study the cell at the molecular level. With the advent of recombinant DNA analysis, refinements in immunohistochemistry and *in situ* hybridization, as well as the introduction of polymerase chain reaction (PCR)-based methodologies, a variety of molecular probe approaches have emerged as potential methods for identifying drug resistance.

There are numerous markers related to specific mechanisms of drug resistance. Measurements of the overexpression of the *MDR1* gene or its product, P-glycoprotein, alterations in the enzymes DNA topoisomerase I and II, changes in the glutathione system (which involve multiple enzymes and isoenzymes), and the overexpression or amplification of oncogenes and their products are but a few of those which have been associated with disease outcome.

Despite the many molecular markers available, there is no clear evidence that their use is superior to cytotoxicity assays for predicting response to chemotherapy. When considering the use of molecular probes to ascertain drug resistance, it must be kept in mind that such resistance may likely be multifactorial. Therefore, tests focused around a specific mechanism of resistance may have limited predictive usefulness. Cell culture-based cytotoxicity assays have the advantage of measuring the net effects of multiple drug resistance mechanisms operating in the cell, whereas the molecular-based assays offer a much more limited assessment.

The development of *in vitro* tests to guide the rational use of chemotherapy has long been a goal in the treatment of cancer. The challenge is to develop laboratory tests that measure levels of the suspected drug resistance genes in clinical samples. The isolation of genes such as *MDR1* provides molecular markers, which offer an alternative to clonogenic assays. However, many uncertainties must be addressed before general screening of drug resistance gene expression can be fully recommended. For instance, what is the best assay to measure gene expression, and can it be standardized for widespread screening? Are measurements to be made at the protein or nucleic acid level? Which gene(s) should be measured? Is the marker of interest expressed in normal tissues? What is the functional significance of the gene in question? Thresholds for clinically significant levels of expression of various drug resistance genes must be established. If elevated levels are found in tumor cells, how does such a finding relate to clinical drug resistance? Can the negative prognosis that its expression confers be altered by the use of alternative treatment strategies?

Critical issues related to tissue sampling strategies must be resolved. For example, are laboratory tests based on biochemical analyses of bulk tissue sufficient, or are more tissue-oriented methodologies such as immunohistopathology or *in situ* hybridization required? Using the scenario of the multidrug resistance phenotype, if very low levels of P-glycoprotein at the cellular level result in clinically significant chemoresistance, then biochemical analyses of bulk tissue specimens using very sensitive methods, such as quantitative PCR, make sense, assuming that the level of expression among tumor cells is relatively uniform. On the other hand, if high-level expression of P-glycoprotein by a small subpopulation of tumor cells more acurately identifies chemoresistant tumors, immunohistochemistry or possibly *in situ* hybridization could emerge as a preferred approach to laboratory testing.

Molecular probe assays should be suitable for testing small numbers of tumor cells and should be sensitive enough to detect small differences in gene expression which are likely to occur in tumor cells with low levels of clinical drug resistance. Northern and Western blotting methods offer the assurance of specificity, but are difficult to apply to all clinical samples as they are time consuming and require large samples (G19). Clinical utility requires a rapid sensitive specific screening method suitable for testing small numbers of tumor cells. Ideally, the method would detect low levels of gene expression and effectively distinguish resistant from sensitive neoplastic cells and neoplastic from reactive host cells.

Tumors found to express the P-glycoprotein have been shown to have a poor prognosis (C8). Salmon *et al.* (S4) established a highly significant correlation

between positive immunohistochemical staining for the P-glycoprotein in primary human tumors and *in vitro* tumor cell resistance, as measured by the clonogenic assay system and tritiated thymidine incorporation. Although P-glycoprotein staining does not provide specific information on sensitivity to individual drugs, this procedure is simpler than *in vitro* drug sensitivity testing and potentially can provide rapid results on patients undergoing routine biopsy procedures. Furthermore, depending on tumor type, *in vitro* growth rates required for sensitivity testing are variable, whereas immunohistochemical tests might be applied routinely. Although the findings of Salmon *et al.* (S4) indicate that positive staining for P-glycoprotein is associated with intrinsic drug resistance in fresh tumor specimens, it is unclear whether P-glycoprotein negativity necessarily predicts for sensitivity and response to cancer chemotherapy. Given that a number of potential mechanisms for multidrug resistance exist, of which the MDR1 phenotype is only the first to be clearly identified, a negative P-glycoprotein finding is not likely to predict drug sensitivity on a consistent basis. Patients whose tumor expresses this protein, however,may become candidates for treatment using chemosensitizers in conjunction with chemotherapy (D3, M20, S22) in an attempt to reverse drug resistance.

Clinicians are interested in determining the values of information on the level of *MDR1* gene expression in the selection of specific chemotherapeutic agents for a given tumor. Approaches to quantifying MDR have been developed based on a variety of techniques. They are generally centered around measuring mRNA or the protein. Quantification of the protein by immunoblotting or immunocytochemistry is more direct but often less sensitive than measuring *MDR* message (C7, R11). Measurement of *MDR1* gene amplification, while of interest to researchers, is not of value as a clinical test, since increased expression of the human *MDR1* gene does not generally require gene amplification and *MDR* gene amplification has not been reported in clinical specimens.

The issue of heterogeneity of *MDR1* expression in individual tumors has important ramifications for the development of laboratory testing strategies. Within some cancers expressing *MDR1* RNA, it is possible that there will be a considerable amount of variability in expression from sample to sample. This may correspond to the variability in immunostaining of tumor cells by anti-P-glycoprotein monoclonal antibodies that has been observed within lesions (W5). Furthermore, specimens are composed of heterogenous populations of cells, including both cancer cells and stromal cells. Some stromal cells, such as fibroblasts, have low *MDR1* RNA levels and therefore may contribute to an underestimation of *MDR1* gene expression. On the other hand, overestimation of Mdr1 levels in tumor cells within bulk tumor specimens may result from Mdr1 coexpression in certain lymphocyte subsets, macrophages, and, at some organ sites, vascular endothelial cells (C10, C26, K13, M4, T4).

6.2. DNA Measurements

While gene amplification commonly occurs in tumor cell lines, it is uncommon in the clinical setting, and therefore the screening of human samples at the DNA level is not a practical approach, as it is for other molecular markers (I6, M14).

6.3. RNA Measurements

Methods have been introduced to measure and quantitate *MDR1* gene expression in humans. The use of nucleic acid probes in Northern and slot blot analyses allows for high sensitivity and signal quantitation. However, unless special care is taken to ensure RNase-free conditions, RNA from tissues will be easily degraded. *MDR1* mRNA has been measured using several different techniques, including Northern and slot blot analyses (F9, G13, K1, K3), *in situ* hybridization (S14), RNase protection assays (G13), and the PCR assays (F18, M24, N5). There is an inverse correlation between *MDR1* mRNA levels and *in vitro* sensitivity to specific chemotherapeutic agents for several human tumors (K3). Northern and slot blot *MDR1* RNA analyses are relative sensitive methodologies and offer the assurance of specificity, but they are difficult to apply to clinical samples, since the assays are time consuming and require relatively large samples. An important limitation of Northern and slot blots is that both of these methodologies require at least 10^8 tumor cells (G15).

In situ hybridization with nucleic acid probes on tissue sections provides direct morphological conformation of the presence of *MDR1* mRNA in tumor cells and permits evaluation of the relationship of *MDR1* mRNA expression to specific locations within organs (C4, R14). However, unless the expression of message in individual cells is relatively high, the results of *in situ* hybridization analyses can be difficult to interpret (G15). Additional drawbacks to *in situ* hybridization include the fact that it is laborious and requires a higher level of technical expertise than can be reasonably expected to exist in many hospital clinical laboratories (F1). It remains to be seen whether expense and technical complexity emerge as deterrents to widespread usage of *in situ* hybridization as a standard *MDR1* mRNA test.

The PCR assay has been demonstrated to be the most sensitive assay for expression of *MDR1* and provides an alternative approach to the identification and quantitation of *MDR1* message. Using gene-specific primers, the PCR amplifies cDNA sequences synthesized from total cellular RNA by reverse transcription and provides a reliable semiquantitative assay for very low levels of *MDR* gene expression. The use of PCR analysis overcomes some of the limitations imposed by small tissue samples. Results obtained with this technology are

essentially in agreement with the tissue-specific distribution of *MDR1* mRNA and P-glycoprotein described by Fojo *et al.* (F9). Although this technology must still be refined with regard to its extreme sensitivity and the difficulty in quantification, it does offer the possibility of detecting *MDR1* RNA in a very small number of tumor cells, and the initial efforts to develop it have shown promise (F18, M24, N5).

To adequately evaluate the issue of heterogeneity, evaluation of *MDR1* expression at the single-cell level may be required. Techniques such as immunohistochemistry or *in situ* hybridization are preferred for detecting *MDR1* expression in specimens in which only a small percentage of cells express P-glycoprotein at detectable levels. The degree of correlation between levels of message and protein remains to be established, since low levels of message can be due to relatively slow rates of transcription or rapid rates of RNA degradation.

6.4. Protein Measurements

The clinically significant level of P-glycoprotein expression in tumor cells is not yet known and may vary among different tumor types. To detect P-glycoprotein expression, Western blotting, immunohistochemistry, and flow cytometry have been used. Western blotting was the first of these methods to be used, but suffers from the same technical limitations as Northern and slot blotting methodologies, that is, the requirement of a large number of cells and the inability to discriminate expression between tumor and nontumor cells (B9, G6). Immunohistochemistry has the advantage of preserving the tissue architecture and detecting signal in individual cells, thus enabling the investigator to ascertain whether expression is observed in a tumor or nontumor.

Several monoclonal antibodies to P-glycoproteins have been developed and used to study surgical pathology and autopsy specimens. The first monoclonal antibody to be described with a high affinity for P-glycoprotein was C219, developed by Ling's group in Toronto (K5). Monoclonal antibody C219 reacts with all known P-glycoprotein isoforms (G5) and is not species specific (C7). It recognizes a small highly conserved C-terminal intracellular epitope located near the nucleotide binding site, which helps to explain its cross-reactivity with both human P-glycoprotein isoforms. The specificity of this antibody for P-glycoproteins has also been controversial, since it has been reported to cross-react with muscle myosin (T4, V5). The possibility that C219 may cross-react with additional proteins also remains an open question. Of the anti-P-glycoprotein monoclonal antibodies introduced to date, C219 has been the most extensively characterized.

Two additional monoclonal antibodies developed by Ling's group (G5, K5) include C494 and C32. Monoclonal antibody C494 is gene specific, binding to a sequence present only in the class I isoforms of hamster and human P-glycoproteins. C32 recognizes a sequence that is conserved in hamster class I and II isoforms but not the class III isoform. Georges *et al.* (G5) used a battery of monoclonal antibodies (C219, C494, and C32) to demonstrate differential expression and organ-specific localizations of the three P-glycoprotein isoforms in hamster tissues.

Another commonly used monoclonal antibody, MRK16, is a human-specific antibody with high affinity for the Mdr1 isoform (H4, T4). MRK16 recognizes an outer surface epitope, and because of this, it can be used to stain living cells. MRK16 must be used on unembedded specimens, whereas C219 can be used on both frozen and paraffin sections.

Another antibody, JSB-I, was developed in The Netherlands and recognizes a highly conserved epitope close to but not overlapping C219. JSB-I was introduced as an immunochemistry reagent for detecting P-glycoprotein in acetone-fixed cells for immunocytochemistry and may be useful in detecting P-glycoprotein in human tumor cells with a low level of drug resistance (B23, S7). Studies comparing C219 and JSB-I immunostaining have produced very similar or identical results (V5, W7).

Additional monoclonal antibodies to P-glycoproteins have been introduced. Murine monoclonals HYB-612 and HYB-241 have been developed by Hybritech (San Diego, CA) in collaboration with the group at Memorial Sloan–Kettering Cancer Center in New York (C25). Monoclonal antibodies UIC2 (M10) and 4E3 (A8) recognize external epitopes of the P-glycoprotein. The clinical utility of these newer monoclonal antibodies remains to be determined.

As discussed in Section 6.1, clinical utility requires a rapid sensitive specific screening method suitable for testing small numbers of tumor cells. Ideally, the method would detect low levels of P-glycoprotein and effectively distinguish resistant from sensitive neoplastic cells and neoplastic from reactive host cells. Immunocytochemical assessment of P-glycoprotein may fulfill these requirements (C7, D3, D4, G19, T3). Studies from the University of Arizona Cancer Center suggest that myeloma may be a particularly instructive disease to study relevant to these methodologic issues (D4). The 8226 human myeloma MDR cell lines provided a "gold" standard to compare clinical samples, to test multiple antibodies for Mdr1 avidity, and to allow determination of fixation and titering conditions. The distinctive myeloma plasma cell was readily visualized microscopically, facilitating single-cell computer-assisted image analysis and densitometry. Cytotoxicity assays allowed comparison of *in vitro* doxorubicin resistance to P-glycoprotein density. Immunoblots using probes to Mdr1 insured specificity. This study indicated that all three monoclonal antibodies to P-glycoprotein which were examined (JSB-I, C219, and MRK16) had favorable detection capabilities and were likely to have clinical utility. Both cell lines and patient samples showed well-defined staining of P-glycoprotein along the plasma membranes. The highly resistant 8226/Dox40 and Dox60 cell lines also showed Golgi stain-

ing, indicating internal cytoplasmic Mdr1 localization. The 8226-sensitive cells were appropriately negative for P-glycoprotein with all three antibodies.

A quantitative immunohistochemical assay for P-glycoprotein expression in clinical samples has been developed at the University of Arizona (D4, G19). In this assay, which was initially used to study patients with refractory myeloma, they found P-glycoprotein-positive cells in seven of 13 patients studied. Immunoperoxidase staining was quantitated by measuring the optical density of individual cells with the CAS 100 (Becton Dickenson Mountain View, CA) optical microscope image analysis system and comparing it to the optical density of the 8226 myeloma cell lines which served as negative and positive standards. This assay system can be used to perform differential counts on tumor cells to determine both the percentage of cells positive for P-glycoprotein and the relative staining intensity of individual cells. Quantitative immunoperoxidase light microscopy may be a useful method for measuring P-glycoprotein expression in clinical specimens.

Antigen fixation has been demonstrated to be a significant variable in P-glycoprotein detection (G19). Both C219 and JSB-I were optimized by fixation in cold acetone. In contrast, optimal results with MRK16 were obtained on unfixed cells or formalin fixation. It is likely that the acetone permeabilizes the membrane, unmasking an internal location for C219 and JSB-I. Of interest, air-drying also appeared to permeabilize the membranes, allowing reactivity with internal epitopes.

Optimized antibody titrations and antigen fixation could prove pivotal in low-level Mdr1 detection. A study of immunocytochemical P-glycoprotein detection in ovarian carcinoma cell lines emphasized the need for overnight fixation and a second-stage "sandwich" to ensure low-level detection of Mdr1 (8-fold multidrug resistance) (C7). In contrast, Grogan *et al.* (G19) found that a short incubation and a single second stage allowed for detection of a 4-fold level of multidrug resistance. Their sensitivity without prolonged incubation or the more elaborate sandwich technique might relate largely to fixation. The study by Chan *et al.* (C7) used mixed formalin–ethanol–acetone fixation, which greatly reduces the signal-to-noise ratio, perhaps necessitating the prolonged incubation and sandwich methods described.

Epitope masking may also be a problem which must be contended with in various clinical samples. Cumber *et al.* (C33) reported that only 12% of samples from patients diagnosed with chronic lymphocytic leukemia were positive for P-glycoprotein expression, a figure which was significantly lower than expected. When the same samples were incubated with neuraminidase to remove sialic acid residues, the proportion of samples positive for P-glycoprotein increased to 52%.

In general, there has been a good correlation between the results obtained with Western blotting and immunohistochemistry in human tumor cell lines. When compared directly, immunohistochemistry has proved to be more sensitive than Western blotting in detecting P-glycoprotein expression (D4, F16, G19).

The "best" assay for measuring *MDR1*/P-glycoprotein expression in human tumor samples has not been established, and while all of the techniques mentioned above are sufficient to ascertain the *MDR* status, they do not address the functionality of the molecule (see Table 4). Therefore, the need exists for assays in which the function of the P-glycoprotein can be assessed.

Several such assays have been developed which rely on the use of flow cytometry in conjunction with fluorescent compounds such as daunomycin or rhodamine-123 (C11, L10, W2). In these assays functionality is generally assessed by measuring the "brightness" of a cell in the presence or absence of P-glycoprotein inhibitors such as verapamil. When rhodamine was compared directly to daunorubicin, it was found to be a more sensitive indicator (L10).

Another approach which has been developed utilizes the synthetic γ-emitting organotechnetium complex [^{99m}Tc]SESTAMIBI, a lipophilic cationic radiopharmaceutical which has been used in cardiac imaging (P7). This compound was found to be a suitable substrate for P-glycoprotein both *in vitro* and *in vivo*. Taking advantage of the γ-emission properties of ^{99m}Tc, Piwnica-Worms *et al.* (P7) were able to demonstrate P-glycoprotein function in a human tumor xenograft model established in the nude mouse.

Whichever method is chosen to assess the P-glycoprotein or *MDR1* status of clinical samples, a critical requirement is the use of consistent standards among the various laboratories carrying out such measurements. The cell lines used as both positive and negative controls must be well characterized and yield consistent results when calibrating the assay. Another very important issue is determin-

TABLE 4
METHODS OF *MDR1*/P-GLYCOPROTEIN DETERMINATION

DNA
Southern blot
RNA
Northern blot
Slot blot
RNase protection assay
In situ hybridization
Reverse transcriptase–polymerase chain reaction
Protein
Western blot
Immunohistochemistry
Flow cytometry

ing exactly what constitutes a positive sample; that is, in the case of immunohistochemistry, what percentage of the cells must stain positive before the sample is considered positive for P-glycoprotein expression? This is an important issue which must be resolved before such measurements can be carried out in a meaningful manner.

7. *MDR1* Expression

7.1. Normal Tissues

The expression of P-glycoprotein in normal human tissues has been studied primarily through the use of immunohistochemical techniques (see Table 5). Within those tissues found to express P-glycoprotein, its expression is localized

TABLE 5
EXPRESSION OF P-GLYCOPROTEIN (P-Gp) IN NORMAL HUMAN ORGANS AND TISSUES[a]

High levels of P-Gp
Adrenal
Intermediate levels of P-Gp
Breasts
Lungs
Gastrointestinal tract
Liver (*MDR2*)
Pancreas
Kidneys
Prostate
Seminal vesicles
Urinary bladder and ureter
Uterus
Placenta
Low levels of P-Gp or nonexpressors
Brain (except capillary endothelia)
Heart
Ovary
Testes (except capillary endothelia)
Tonsils
Spleen
Thymus
Bone marrow
Skeletal muscle
Smooth muscle
Skin

[a]Adapted from Refs. C27 and W5.

typically to the apical surface of the cells. High levels of expression have been found in human adrenal cortical cells, the brush border of the renal proximal tubule epithelium, the luminal surface of biliary hepatocytes, small and large intestinal mucosal cells, and pancreatic ductules (B1, C25, C27, C32, F9, M23, R8, S25, T3, W5). P-glycoprotein has also been found to be expressed at lower levels in capillary endothelial cells of the brain and the testis (C26, T4), placenta (C27), lung (C27), prostate (C27), stomach (C27), natural killer cells (K13, W8), and $CD34^+$ bone marrow stem cells (C11).

The polarized localization of *MDR1* at apical (luminal) epithelial surfaces suggests a role in transport (A9, G16, P1). It has been proposed that P-glycoprotein has a role in the secretion of naturally occurring lipophilic substances and provides a protective mechanism against cytotoxic plant alkaloids and other dietary xenobiotic substances (R15). This idea is supported by studies in which the *MDR1* cDNA was expressed in monolayer kidney epithelial cells grown on filters in which a basal-to-apical transport of various drugs and other agents was demonstrated (H14, H16, P2). While this hypothesis may be applicable in organs such as the colon, in others it is not. For example, P-glycoprotein levels are high in the normal adrenal gland, where it is a cytoplasmic component rather than a plasma membrane component. Here, it may function as an intracellular transporter for steroids (Q1, U5, V6, Y2). Increased *MDR1* gene expression has been demonstrated in the mouse uterus during pregnancy (A6), and the highly hydrophobic sterol, progesterone, has been shown to interact directly with it (Q1).

Expression in normal capillary endothelial cells is relevant, since this may explain blood–brain- and blood–testis-type barriers that affect drug levels attainable in the central nervous system and the testis (C26, T1). The blood–brain barrier is maintained primarily by capillary endothelial cells with continuous intracellular tight junctions and specific plasma membrane transport systems (B22). Studies by Tsuji *et al.* (T9) and Hegmann *et al.* (H7) have shown that rhodamine-123 and vincristine are actively transported out of these cells, demonstrating that the P-glycoprotein expressed is functional. In the brain and the testis P-glycoprotein may thus serve to keep toxic metabolites and xenobiotics out of the tissues. *MDR1* expression in subsets of lymphocytes is potentially relevant for several reasons. First, lymphocyte P-glycoprotein per se may contribute to the multidrug resistance phenomenon in certain tumors. And second, P-glycoprotein-positive lymphocytes as well as other *MDR1*-expressing stromal cells and normal epithelial cells complicate the quantitation of the protein, or its message, in bulk tissue samples (see above). Interestingly, *MDR1* expression in normal granulocytes was not associated with the transport of rhodamine-123 out of the cell (K13). What emerges is the concept that Mdr1 is involved in diverse and possibly unrelated physiological processes in different normal organs. A functional role for the Mdr2 isoform remains to be delineated.

Two obstacles to determining the site-specific distributions of P-glycoprotein

isoforms in human tissues are the variability and heterogeneity of *MDR1* expression (W4). Variability is defined as differences in the level of expression of *MDR1* or P-glycoprotein among individuals, while heterogeneity is defined as the differences in levels of expression of these markers within an organ or tumor. Fojo *et al.* (F9) first demonstrated a broad range of *MDR1* mRNA levels in the "normal" human colon adjacent to a tumor in eight patients. Similar studies on *MDR1* mRNA are needed on the normal human colon and other normal organs from patients without tumors or other organ pathology. Both variability and heterogeneity of anti-P-glycoprotein staining by immunohistochemistry seem to be the rule rather than the exception (W4).

7.2. Tumor Expression

Studies of human tumor samples have suggested a role for the *MDR1* gene in both intrinsic and acquired drug resistance. Intrinsic multidrug resistance generally occurs in tumors arising in tissues that normally express P-glycoprotein. Organs in which relatively high levels of *MDR1* expression occur tend to give rise to higher percentages of tumors that express P-glycoprotein.

The first studies on P-glycoprotein expression in human cancer patients were reported in the 1980s. Bell *et al.* (B9) were the first to report P-glycoprotein expression in human tumor specimens. Using Western blotting analysis, they reported that two of five ovarian carcinoma samples expressed the P-glycoprotein. Gerlach *et al.* (G6) surveyed a series of human solid tumors from 46 patients, representing 12 tumor types, for evidence of P-glycoprotein expression and found overexpression in four of 11 sarcomas by immunoblot analysis.

In another study the *MDR1* gene expression in human tumors and normal tissues was assessed using probes prepared from *MDR1* cDNA in conjunction with Northern and slot blot analyses (F8, F9). High levels of the message were detected in several human tumors, including some but not all tumors of epithelial origin arising in the adrenal gland and the colon. In a comparison of primary and recurrent tumors, increased expression of *MDR1* mRNA was found following chemotherapy (F9).

Malignancies that were initially sensitive to chemotherapy have been studied at relapse. Goldstein *et al.* (G12) observed increased levels of *MDR1* RNA in several patients who had initially responded to chemotherapy and subsequently relapsed. Others have confirmed this observation of increased levels of *MDR1* expression in relapsing patients (G21, M5, P5).

P-glycoprotein expression is a poor prognostic factor. Chan *et al.* (C8) found a significant correlation between P-glycoprotein expression and outcome in childhood sarcomas. Both the disease-free survival and the overall survival were significantly reduced in the group whose tumors were positive for P-glycoprotein, while other prognostic factors such as age, pretreatment lymphocyte counts,

size of the tumor, or unfavorable histology were not significant different. Salmon *et al.* (S4) established a highly significant correlation between positive immunohistochemical staining for P-glycoprotein in primary human myeloma, lymphoma, and breast tumors and *in vitro* tumor cell resistance, as measured by the clonogenic assay system and tritiated thymidine incorporation. They examined 26 patients who were either previously untreated or were in relapse following chemotherapy and found that 12 of the 26 cases expressed P-glycoprotein. All 12 were found to be resistant to doxorubicin in the *in vitro* assays.

In addition to its role in anticancer drug resistance, enrichment of cancer cells with P-glycoprotein may alter their biological behavior. Weinstein *et al.* (W6) studied the relationship of P-glycoprotein expression in invasive colon carcinomas to tumor dissemination in a series of 95 primary colon carcinomas. They found a strong association between the presence of anti-P-glycoprotein monoclonal antibody reactivity in invading tumor cells at the leading edge of the tumor and both vessel invasion ($P < 0.001$) and lymph node metastases ($P < 0.01$) (W6). The mechanistic basis of this phenomenon remains to be elucidated. It has been suggested that the insertion of P-glycoprotein into the plasma membrane influences cell locomotion (W6) and intercellular adhesion (G20), both of which could result in the enhancement of tumor aggressiveness by P-glycoprotein. Thus, P-glycoprotein expression may represent a double-edged sword by endowing tumor cells with a means for resisting certain forms of anticancer therapy as well as facilitating their dissemination.

7.2.1. *Solid Tumors*

The general incidence of P-glycoprotein expression in solid tumors varies widely among different tumor types. In 1989 Goldstein *et al.* (G13) published the largest study to date of *MDR1* mRNA expression in human tumors. Using slot blot hybridization and RNase protection assays, they analyzed over 400 bulk tumor samples representing a wide spectrum of human malignancies. Tumors arising from organs with P-glycoprotein as a normal epithelial component had relatively high levels of *MDR1* mRNA expression and were generally resistant to chemotherapy. These tumors included colon cancer, renal cell carcinoma, hepatoma, adrenocortical carcinoma, pheochromocytoma, islet cell tumors of the pancreas, carcinoid tumors, and non-small-cell carcinoma with neuroendocrine properties. In a study of 11 patients with adrenocortical carcinoma, Flynn *et al.* (F5) found a 100% incidence of P-glycoprotein expression using a panel of four monoclonal antibodies. They reported no correlation between P-glycoprotein expression and tumor grade, stage of disease, or survival, however.

P-glycoprotein expression has been reported to be elevated in 40% of newly diagnosed pediatric primitive neuroendocrine tumors, another highly drug-resistant tumor (T7). Other tumors, such as neuroblastoma and astrocytoma, were found to have occasionally high or intermediate levels of *MDR1* mRNA

expression, and recurrent tumors in patients who developed resistance to chemotherapy tended to have higher levels of message than initial tumors from the same patients (G13). Goldstein *et al.* (G12) analyzed 49 neuroblastoma samples using a slot blot technique and found that the majority of both treated and untreated patients expressed detectable levels of *MDR1* RNA. They reported that five of 18 treated patients expressed high levels, while three of 31 untreated patients expressed high levels.

MDR1 expression was found to be high in both colorectal and gastric carcinomas (W6) as well as in adenocarcinomas arising in Barrett's esophagus (R9). Weinstein *et al.* (W6) found that 65 of 95 primary colon adenocarcinomas which were Duke's stage B1 or greater were P-glycoprotein positive by immunohistochemistry. None of these patients had received prior chemotherapy. Such findings reflect the high levels of P-glycoprotein normally found in these tissues.

P-glycoprotein has also been implicated in cervical carcinoma. Schneider *et al.* (S8) reported that 10 of 11 cervical carcinomas, including two which had previously received chemotherapy, expressed P-glycoprotein as detected by immunohistochemistry.

The question of P-glycoprotein expression in breast cancer remains problematic. Sugawara *et al.* (S25) detected P-glycoprotein expression in breast cancer specimens using monoclonal antibody MRK16, but in a larger study, Merkel *et al.* (M13) failed to detect *MDR1* expression in over 248 cases of breast cancer, many of whom had received prior chemotherapy containing MDR-related agents. Dixon *et al.* (D16) also failed to demonstrate P-glycoprotein staining in 26 patients with locally advanced breast cancer. In nine of 57 breast cancer specimens, two of which had received prior chemotherapy, Goldstein *et al.* (G13) reported increased RNA levels. Verrelle *et al.* (V7) found a high level of P-glycoprotein in breast cancer. Using monoclonal antibody C494, they found that 17 of 20 untreated locally advanced breast cancer cases were positive. Patients whose tumors were positive had a shorter period of disease-free survival. Ro *et al.* (R8) also observed a high level of expression. They observed that 20 of 40 breast cancer specimens were positive for P-glycoprotein at the time of mastectomy, and there was no correlation between P-glycoprotein expression and patient age, number of involved lymph nodes, clinical stage, or steroid receptor content. In a smaller series of breast cancer patients, Salmon *et al.* (S4) reported that five of 13 were positive for P-glycoprotein expression, four of the five having received prior chemotherapy. All five of the positive samples were found to be resistant to doxorubicin in an *in vitro* assay.

MDR1 mRNA has not been detected in all tumors, particularly not in carcinomas arising in organs that are normally low expressors of P-glycoprotein. These include untreated breast cancer, both non-small-cell and small-cell lung cancers, and bladder cancer. Also in this group are head and neck cancer, melanoma, mesothelioma, ovarian cancer, prostate cancer, sarcoma, and Wilms' tumor

(G13). In malignant melanoma and non-small-cell lung carcinoma, in which the response to chemotherapy is very limited, no elevation in *MDR1* expression was observed, providing evidence for the existence of other clinically important mechanisms of drug resistance. Cordon-cardo (C25) reported that only two of 22 lung cancers expressed P-glycoprotein, while Lai *et al.* (L1) reported a low incidence in both non-small-cell and small-cell lung cancers. It has been suggested that expression of the *MDR1* gene may correlate to some extent with higher levels of differentiation in carcinomas (K3).

7.2.2. *Hematological Tumors*

Mdr1 and *MDR1* mRNA levels have been measured in hematopoietic dyscrasias by various groups (H12, I6). In one early study P-glycoprotein was detected in two cases of refractory acute nonlymphoblastic leukemia (ANLL), using an immunocytochemical assay (M1). It was noted in this study that the percentage of peripheral blood cells staining with anti-P-glycoprotein antibodies increased with subsequent treatment. In another study using an *MDR1* gene probe, increased *MDR1* mRNA expression was found in one of 10 patients with acute lymphoblastic leukemia (F9). Ito *et al.* (I6) used immunocytochemistry and Southern and Northern hybridization analyses in a study of 19 cases of adult acute leukemia. They found that P-glycoprotein expression and *MDR1* gene amplification occurred infrequently in leukemic cells at the time of initial presentation as well as at relapse, and thus concluded that classic multidrug resistance cannot account for the refractoriness to antileukemic drugs in most adult patients with acute leukemia. Others have found evidence for increased expression, however.

Holmes *et al.* (H12) screened peripheral blood or bone marrow from patients with myelodysplastic syndromes and acute myeloblastic leukemia and found increased *MDR1* mRNA in 18 of 40 patients. Low-level expression of the *MDR1* gene may be demonstrated in larger percentages of leukemia patients by PCR analysis (R10).

In 63 patients with newly diagnosed acute myeloid leukemia (AML), Pirker *et al.* (P6) found that 71% were positive for *MDR1* gene expression. These patients had a 53% complete response rate, significantly lower than the 89% response rate observed in the *MDR1*-negative group. Seventy-one percent of patients in the positive group died during the 14-month observation period, while only 22% died from the negative group. Both the disease-free survival and overall survival were significantly higher for the negative group.

Adult acute lymphocytic leukemia (ALL), adult nonlymphocytic leukemia (ANLL), non-Hodgkin's lymphoma, and chronic myelogenous leukemia (CML) in blast crisis were found to have occasionally high or intermediate levels of *MDR1* mRNA expression (G13).

Marie *et al.* (M5) studied a series of 41 adult patients with acute leukemias,

which included five cases of ALL, 23 cases of AML, and 13 secondary leukemias. They reported high levels of *MDR1* expression in 50% of the patients who had received prior chemotherapy. In contrast, only 19% of previously untreated patients expressed *MDR1*. Serial determinations were carried out on four cases and an increase in *MDR1* expression was observed in two of the four.

In a series of 36 children and 23 adults with ALL, there was a higher rate of relapse and a decreased overall survival among P-glycoprotein-positive cases in both patient groups (G10). Multivariate analysis demonstrated that these findings were independent of age, immunophenotype, or tumor karyotype.

Michieli *et al.* (M16) examined P-glycoprotein expression in 59 cases of ANLL and found it to be elevated in relapsed patients compared to the time of diagnosis. The failure of front-line therapy, which included daunorubicin, was associated with a progressive increase in P-glycoprotein expression. In a larger study Campos *et al.* (C2) examined 150 patients with newly diagnosed ANLL and reported that leukemias arising from previous myelodysplasia syndromes or induced by therapy were frequently positive by immunohistochemistry. They defined a positive case as one in which, >20% of the leukemia cells were stained by monoclonal antibody MRK16. Responses to chemotherapy were reported to be significantly lower in those patients whose tumors were P-glycoprotein positive (32% complete response rate) than in those who were negative (81% complete response rate). There was also an association between P-glycoprotein expression and $CD34^+$ expression. They concluded that P-glycoprotein is an important prognostic indicator in ANLL.

Tsuruo *et al.* (T15) found that 50% of patients with CML in blast crisis expressed increased levels of both P-glycoprotein and *MDR1* RNA levels. Kuwazuru *et al.* (K17) also reported increased levels from CML patients in blast crisis. They observed that the levels increased at the time of relapse and that those patients whose tumors were positive rarely responded to chemotherapy.

Studying chronic lymphocytic leukemia (CLL), Holmes *et al.* (H13) found increased *MDR1* expression in 18 of 34 patients, including 14 who had received prior chemotherapy. Sequential analysis of several of these patients suggested that the level of expression increased in response to chemotherapy and fell to basal levels when therapy was stopped. Ludescher *et al.* (L9) examined both *MDR1* expression and function in a series of CLL patients. They studied 42 consecutive patients, 23 of whom had received prior chemotherapy, by flow cytometry, using rhodamine-123 to assess P-glycoprotein function. Eighty-one percent of the patients had a marked decrease in rhodamine accumulation. While this decrease was independent of prior treatment status, patients who received prior chemotherapy which included at least one drug associated with the MDR phenotype had a higher percentage of rhodamine-negative cells. *MDR1* gene expression was assessed by PCR analysis and was found to be elevated in 25 of 26 cases examined. Expression of *MDR1* was significantly correlated with rhodamine efflux and neither was correlated with the disease stage, lymphocyte count, or duration of disease.

Myelodysplastic syndromes are characterized by a relative resistance to chemotherapy (G1). Several groups have now demonstrated increased *MDR1*/P-glycoprotein expression in myelodysplasia (H12, L6, S23). List *et al.* (L6) reported a significant association between P-glycoprotein expression and $CD34^+$ expression, a finding that has been confirmed by others (S23). $CD34^+$ cells represent an immature stem cell phenotype and may portend a higher risk of leukemic transformation (S23).

Multiple myeloma is characterized by a high initial response rate to chemotherapy and the eventual emergence of acquired drug resistance (D4). Ultimately, this renders this disease incurable (K18). Several clinical studies have established that P-glycoprotein is expressed in myeloma patients with clinical evidence of drug resistance (D3, D4, E3, S4).

Grogan *et al.* (G21) have demonstrated a strong correlation in multiple myeloma patients between P-glycoprotein expression and prior chemotherapy with vincristine or doxorubicin. They studied a series of 106 consecutive bone marrow specimens from 104 myeloma patients. Myeloma patients with no prior chemotherapy had a low incidence of P-glycoprotein expression (6%), while those receiving chemotherapy had a significantly higher incidence of P-glycoprotein positivity (43%). When the total dose of vincristine surpassed 20 mg, expression increased to 50%, and when doxorubicin exceeded 340 mg, P-glycoprotein expression was observed in 83%. When patients received both high vincristine and doxorubicin dosages, the incidence of P-glycoprotein expression was 100%. Disease duration was not a significant variable, nor did P-glycoprotein correlate with immunophenotypic or other clinical factors.

Among lymphomas detectable levels of P-glycoprotein is uncommon (2%) in untreated patients and frequent (64%) in those with clinically drug-resistant disease (M20). These findings are concordant with the high response rates of lymphomas to initial chemotherapy (75–95%, depending on the disease stage) and the substantially lower response rate in recurrent disease. Several reports indicate that the presence of P-glycoprotein in malignant lymphomas is associated with poor response to therapy (D5, N2, P4). Recent clinical data suggest that P-glycoprotein-positive lymphoma patients benefit from alternative supplemental therapy with chemosensitizers (e.g., verapamil, quinine, and cyclosporine A), which may competitively bind P-glycoprotein and reverse the efflux pump effect (M20). In particular, among 18 patients with drug-refractory lymphoma, 72% responded to standard chemotherapy plus the added P-glycoprotein chemosensitizers, suggesting a benefit for this alternative therapy in carefully selected lymphoma patients with clinical evidence of multidrug resistance and detectable P-glycoprotein (M20). This suggests that P-glycoprotein is an important object of clinical immunophenotypic assay among lymphoma patients.

Thus, it is now well established that, while not detectable in all cases, MDR cells are clinically detectable in many different human cancers and may prove to be an important prognostic factor.

8. *MDR1* Modulation

One of the goals of drug resistance studies is to find a means of circumventing drug resistance which is applicable to the clinical setting. Strategies to overcome *MDR1*-mediated drug resistance include the following: (1) the use of non-cross-resistant chemotherapeutic drug regimens, (2) high-dose chemotherapy as used in conjunction with bone marrow transplantation, (3) targeting *MDR1* with monoclonal antibodies or monoclonal antibody conjugates, and (4) use of chemosensitizing agents which inhibit *MDR1*-mediated efflux.

8.1. Non-Cross-Resistant Chemotherapy

The use of non-cross-resistant therapeutic regimens is perhaps best illustrated in regimens used against Hodgkin's disease. In this example the standard therapeutic regimen of mechlorethamine, vincristine, procarbazine, and prednisone is alternated with a second regimen consisting of doxorubicin, bleomycin, vinblastine, and dacarbazine (B19, D14). The goal of such regimens is to utilize the largest number of active agents at the highest doses possible, assuming that mutations conferring drug resistance will not convey resistance to all of the agents in the regimen.

8.2. High-Dose Chemotherapy

High-dose chemotherapy (or radiation therapy) in conjunction with bone marrow transplantation, such as that used in chronic myelogenous leukemia and malignant lymphomas, represents another approach in overcoming drug resistance. This assumes that, despite resistance to standard doses of antineoplastic agents, a dose–response relationship still exists for these tumors and that high doses of chemotherapy might overcome this resistance. A different strategy, which may prove useful against solid tumors, is insertion of the *MDR1* gene itself into normal human bone marrow cells, using a retroviral vector (M9). Because myelotoxicity is the usual dose-limiting toxicity associated with many chemotherapeutic drugs, rendering the normal bone marrow resistant to the toxic effects may enable patients to tolerate higher therapeutic doses of the drugs. Animal studies support this approach.

8.3. Monoclonal Antibody-Directed Therapy

Another strategy for overcoming drug resistance due to P-glycoprotein is to specifically target overexpressing cells with monoclonal antibodies. MRK16, a monoclonal antibody developed against an external epitope of the P-glycoprotein, has been shown to inhibit tumor formation and reduce tumor volume when administered to nude mice bearing multidrug-resistant human ovarian cancer xenografts (T10). It has also been demonstrated that *Pseudomonas* exotoxin can be conjugated with MRK16, and that this fusion protein kills multidrug-resistant cells in tissue culture (F4). The MRK16–*Pseudomonas* exotoxin conjugate was shown to specifically target and result in a dose-dependent killing of *MDR1*-expressing human renal carcinoma cell lines, while not affecting non-*MDR1*-expressing human tumor cell lines (M18). When the conjugate was administered to transgenic mice whose bone marrow cells express the human *MDR1* gene product, there was a dose-dependent decrease in white blood cells, while in normal mice there was no such decrease, indicating that the observed cytotoxicity in the transgenic mice was specific for the human *MDR1*-expressing cells. Caution should be taken when interpreting these results, however. The MRK16 antibody is specific for the human P-glycoprotein and therefore may target *MDR1*-expressing cells in normal tissues as well as in tumor cells, thus potentially leading to unacceptable toxicities. Reagents such as this could, however, be utilized for the *ex vivo* purging of *MDR1*-expressing cells from the bone marrow and therefore warrant further development. Recently, several new monoclonal antibodies have been introduced. Monoclonal antibodies UIC2 and 4E3 both recognize external epitopes of the human P-glycoprotein and may present new therapeutic opportunities (A8, M10).

8.4. Chemosensitization

An area receiving considerable attention in the laboratory, and more recently in the clinic, has been the use of chemosensitizers to reverse *MDR1*-mediated drug resistance. This work is based on the observations by Tsuruo *et al.* (T13, T14), who reported that certain compounds, including the calcium channel blocker verapamil, were able to reverse vincristine resistance in murine leukemia cells. This opened the door for the development of new strategies to overcome P-glycoprotein-mediated MDR. Agents such as verapamil or members of the other classes of compounds that can function as inhibitors of P-glycoprotein have been effective in reversing drug resistance in model systems (B8).

8.4.1. *In Vitro*

Beginning with the work of Tsuruo *et al.* (T13, T14), demonstrating that verapamil could reverse drug resistance, an increasing number of agents have

been identified that can successfully reverse multidrug resistance *in vitro*. To date a wide variety of compounds representing several different drug classes have been shown to modulate P-glycoprotein-mediated MDR *in vitro*. Included among these agents are the calcium channel blockers (B11, H5, R3, T13, T14), calmodulin inhibitors (A1, F12, F14, G2, G3, H1), surfactants (C24, R7, W12, W13), anthracycline analogs (S10, S17), steroids and their derivatives (F15, R2, Y2), antimalarials (A1, I5, S15, T11, Z2), cardiovascular drugs (A10, C13, S11, S15, T11), antibiotics (A1, G14, H11, S15), and immunosuppressants (A7, C5, H2, K8, L8, S18, T16, T17) (see Table 6). For a more thorough discussion of these compounds, the reader is referred to two recent reviews on the subject (B5, F13). Akiyama *et al.* (A1) studied a large series of compounds which modulate MDR by examining their ability to inhibit the binding of a photoaffinity-labeled vinblastine analog to the P-glycoprotein. Agents such as reserpine, quinidine, and cepharanthine were able to inhibit binding in concentrations which were comparable to those required to reverse multidrug resistance *in vitro*. Other compounds such as chloroquine, propranolol, or atropine, which only partially reverse multidrug resistance, had no effect on the binding of the vinblastine analog to the P-glycoprotein. Interestingly, agents such as the calmodulin inhibitors chlorpromazine and trifluoperazine, which are effective in completely reversing MDR *in vitro*, were poor inhibitors of photoaffinity labeling of the P-glycoprotein. Such findings suggest that the compounds which modulate multidrug resistance may not operate by binding the P-glycoprotein directly. Although the mechanism of reversal of MDR by these agents is not fully understood, it likely involves the alteration of drug transport by competitive binding to P-glycoprotein and/or altering intracellular binding of chemotherapeutic agents.

TABLE 6
AGENTS THAT REVERSE MULTIDRUG RESISTANCE

Class	Example	Reference
Anthracycline analogs	Cyanomorpholino-doxorubicin, *N*-acetyldaunorubicin	S10, S17
Antibiotics	Cepharanthidine, cefoperazone, erythromycin	A1, G14, H11, S15
Antimalarials	Chloroquine, quinidine, quinine	A1, I5, S15, T11, Z2
Calcium channel blockers	Verapamil, nicardapine, diltiazem	B11, H5, R3, T13, T14
Calmodulin inhibitors	Chlorpromazine, trifluoperazine	A1, F12, F14, G2, G3, H1
Cardiovascular drugs	Propranolol, amiodarone, dipyridimole	A10, C13, S11, S15, T11
Immunosuppressants	Cyclosporine A, FK 506, PSC-833	A7, C5, H2, K8, L8, S18, T16, T17
Steroids and derivatives	Progesterone, tamoxifen	F15, R2, Y2
Surfactants	Tween 80, Solutol HS-15 (BASE Corp., Cambridge, MA)	C24, R7, W12, W13

Agents such as verapamil may not, however, be limited to a single mechanism of action in reversing multidrug resistance. For example, Safa *et al.* (S2), utilizing a photoaffinity analog of azidopine, demonstrated that this compound binds to the P-glycoprotein in MDR cells and that verapamil can displace a vinblastine analog from this site. Verapamil has also been shown to concentrate in lysosomes, and indeed various other lysosomotropic amines are known to reverse multidrug resistance (S15, Z2). It has also been shown to alter the subcellular distribution of daunorubicin in HL-60 human promyelocytic leukemia cells which exhibit the MDR phenotype (H10).

Cyclosporine A, a widely used immunosuppressant, appears to be one of the most potent modulators of MDR *in vitro*. It has been demonstrated to enhance the cytotoxicity of MDR-related agents, including doxorubicin and vincristine, in both murine and human MDR cell lines (C5, H2, S18). At clinically achievable concentrations, cyclosporine A completely reversed doxorubicin resistance in an ovarian cancer cell line (C5). The cyclosporines differ from other modulators of P-glycoprotein-mediated MDR in that they have been demonstrated to modulate non-MDR as well as MDR cell lines (H2, S18). FK 506, a newly characterized immunosuppressant, has also been demonstrated to modulate P-glycoprotein-mediated MDR *in vitro* (A7).

Because of its immunosuppressive and nephrotoxic effects, cyclosporine A may be of limited use in the clinical setting. Nonimmunosuppressive cyclosporine analogs devoid of toxic renal effects are currently undergoing study. One of the first to be studied has been PSC-833, an analog of cyclosporine D. It was shown to be 7- to 10-fold more potent than cyclosporine A in reversing vincristine and doxorubicin resistance in MDR cell lines (K8, T17). Additional analogs being studied include SDZ 280-446, a nonimmunosuppressive hydrophobic peptide which has been shown to be as potent as PSC-833 in reversing MDR (L8).

8.4.2. *Structure–Activity Studies*

Several studies have focused on elucidating the structure–activity relationship of the various MDR chemosensitizers in an effort to arrive at the ideal structural requirements. Zamora *et al.* (Z3), studying a series of indole alkaloids known to reverse P-glycoprotein-mediated MDR, developed a set of "rules" for the design of an ideal reversal agent. They found that the most potent chemosensitizers were hydrophobic molecules with two planar aromatic rings and a tertiary basic nitrogen which was charged at physiological pH. While many of the known MDR chemosensitizers fit such a profile, there are exceptions, most notably cyclosporine A.

A study of various phenothiazine analogs revealed a number of alterations which affected the activity of these compounds (F14). It was found that substitutions to the phenothiazine ring which increased hydrophobicity also increased

their potency as chemosensitizers. Compounds with a four-carbon bridge rather than a two- or three-carbon bridge were more active, as were analogs with a piperazinyl amine rather than a noncyclic amine. Tertiary amines were more potent than primary or secondary amines. Thus, they concluded that there were three basic components which affected activity: the hydrophobicity of the tricyclic ring, the length of the alkyl bridge, and the charge of the terminal amino group. The most potent phenothiazine analog was found to be (*trans*)-flupenthixol, which was 2–7 times more potent than the *cis* isomer (F12).

Using a novel approach, Klopman *et al.* (K14) studied the structure–activity relationship of a diverse group of chemicals known to reverse MDR, utilizing an artificial intelligence computer program known as MULTICASE to delineate common structural features of these compounds, which they referred to as "biophores." Using the predicted biophores, they identified seven new compounds, four of which displayed substantial activity against multidrug-resistant CHO cells *in vitro*.

Other studies have been carried out with dihydropyridines (N4), dipyridamole (A10), and phenoxazines (T5), but the question as to what represents the ideal chemomodulator remains unanswered.

8.4.3. *In Vivo*

The *in vivo* efficacy of MDR chemosensitizers has not been as extensively studied. In general, the models used employ mice inoculated intraperitoneally with either human or murine MDR cell lines derived *in vitro*. The mice are then administered chemotherapy alone or with the MDR modulator. Drug administration has been achieved by a number of routes and methods, but in general the intraperitoneal route has been utilized for many of the studies. The primary end point has been a measure of the life span of the animals treated with the combination of chemotherapeutic agent and chemosensitizer compared with that of animals treated with the chemotherapeutic agent alone. Modulators of MDR, such as verapamil (R1, T12, T13), quinidine (T11, W11), and cyclosporine A and its analogs (B18, K7, L8), retain their activity in these *in vivo* models. In the *in vivo* setting compounds such as verapamil have been shown to increase survival time by 40–50%, while agents such as cyclosporine A and PSC-833 enhance survival by >200% when compared to control animals (B18, T13).

Recently, several new animal models have been introduced. Mickisch *et al.* (M17) have developed a transgenic mouse in which the bone marrow cells have been transfected with the human *MDR1* gene. In this model the measurement of white blood cell counts provides a simple and reliable means of assessing the potency of MDR-reversing agents (M17, M18). Unfortunately, though, the model does not directly assess the ability of these compounds to eradicate the tumor population.

We have established a reproducible *in vivo* model of human multiple myeloma in the severe combined immunodeficient (SCID) mouse using both the RPMI 8226 human myeloma cell line and the P-glycoprotein-expressing multidrug-resistant 8226/C1N subline (B12). The SCID mouse is well suited as a model for myeloma because (1) human–SCID xenografts are readily attained, (2) xenografts achieve consistent growth and spread over a short period, (3) tumor cells are readily detected by their immunoglobulin secretion, (4) the amount of immunoglobulin secreted is proportional to the tumor burden, thereby facilitating calculation of therapy effects, and (5) differential therapy effects in drug-sensitive versus drug-resistant cell lines are readily demonstrable.

When SCID mice were injected intraperitoneally with either 8226 drug-sensitive or P-glycoprotein-expressing multidrug-resistant myeloma cells (8226/C1N), tumors were detected within 5 days after injection by the presence of human λ light chain excretion in the mouse urine. The antineoplastic agent doxorubicin was effective in treating the drug-sensitive 8226 human–SCID xenografts, but had no effect on the multidrug-resistant 8226/C1N human–SCID xenografts. In the 8226-sensitive xenografts treatment with doxorubicin resulted in a sharp decline in the concentration of human λ light chain excreted in the mouse urine. This correlated with an increased survival of the drug-treated animals. The initial evaluation of chemosensitizers in this model has been performed using verapamil. The combination of verapamil and doxorubicin resulted in both a decrease in light chain excretion and an increase in the survival of those animals bearing the 8226/C1N tumor (B13).

This model can be used to identify the limiting organ-specific toxicities of new chemomodulators both when administered alone and in combination with chemotherapeutic agents. The use of clinically relevant animal models is necessary because many new MDR chemosensitizers, either single agents or combinations, while appearing promising *in vitro*, may be inactive or prohibitively toxic in patients.

8.5. Clinical Studies

Pilot clinical studies using verapamil or cyclosporine A as chemosensitizers have been conducted with myeloma, lymphoma, and leukemia patients (D3, M20, S22) and have supported the use of MDR chemosensitizers in additional trials.

8.5.1. *Verapamil*

After the demonstration of its ability to reverse MDR *in vitro*, verapamil was the first modulator studied clinically. Benson *et al.* (B14) reported the preliminary findings of a phase I clinical study investigating the combination of vin-

blastine and verapamil in 17 patients with solid tumors. There was no augmentation of vinblastine toxicity by verapamil, and the dose-limiting toxicities observed were those due to verapamil: Electrocardiographic changes representing first-degree heart block, junctional rhythms, and nonspecific T-wave changes. In a phase I study of oral verapamil and doxorubicin in 13 patients with drug-refractory tumors, which included colon, breast, and pancreatic carcinomas, Presant *et al.* (P8) reported cardiac toxicities associated with doses above 120 mg every 6 hours. They observed a partial response in one patient lasting 8 weeks.

In a phase I/II study Ozols *et al.* (O2) failed to show that intravenous verapamil combined with doxorubicin had an effect in reversing drug resistance in eight patients with refractory ovarian cancer. All eight had received prior chemotherapy with alkylating agents, but none had received doxorubicin. Verapamil was administered as an intravenous bolus followed by continuous infusion. The dose was escalated until a maximum tolerated dose of verapamil was established, as defined by hypotension or heart block, and then maintained for 72 hours. This study did not assess the presence or absence of P-glycoprotein in the patient samples.

The group at the University of Arizona has reported the most extensive experience with verapamil in the setting of multiple myeloma (D3, D4, P3, S3). In these studies verapamil was added by continuous intravenous infusion to the vincristine–doxorubicin–dexamethasone (VAD) regimen at the time of relapse. All patients had progressive disease while on the VAD regimen and P-glycoprotein expression assessed by immunohistochemistry. The response rate in P-glycoprotein-positive patients placed on VAD–verapamil was 40% (S3). The duration of response has, unfortunately, been short to date. The dose-limiting toxicities associated with the addition of verapamil were hypotension and cardiac arrhythmias (P3).

Miller *et al.* (M20) reported their experience with verapamil in lymphoma patients. Using immunohistochemical assessment of P-glycoprotein expression, they reported that 2% of newly diagnosed and 64% of previously treated patients had tumors that were positive. In this study patients received a 5-day continuous infusion of verapamil in combination with cyclophosphamide (C)VAD if they had failed or relapsed within 3 months of treatment with doxorubicin and vincristine. A response was achieved in 13 (72%) of patients, with a complete response observed in five (28%).

A major problem associated with the use of verapamil is that the concentrations required to reverse multidrug resistance *in vitro* are at or above the threshold of serious verapamil clinical toxicities (P3). Clearly, while proving a point of principle, verapamil is not an ideal MDR-reversing agent. Newer, more potent, and less toxic agents are needed. Studies are now under way to evaluate oral

R-verapamil (the +-enantiomer), which is less cardiotoxic than and equally effective as racemic verapamil in modulating MDR *in vitro*.

Thus, studies have indicated that the cardiovascular toxicities of verapamil were dose limiting, and that systemic concentrations produced were not sufficient to reverse MDR completely. Despite these limitations results with verapamil have been encouraging, particularly for hematological malignancies.

8.5.2. *Cyclosporine A*

Cyclosporine A is the chemosensitizer which has received the most attention to date as a result of its *in vitro* potency. In 72 patients with a variety of tumors, Yahanda *et al.* (Y1) conducted a phase I study of cyclosporine A and VP-16. Cyclosporine was administered as a continuous infusion at a rate of 18 mg/kg/day following a loading dose of 6 mg/kg, with plasma levels of >2000 μg/ml being achieved. The major toxicity associated with this treatment was a transient hyperbilirubinemia which was observed in 54% of the patients. Other toxicities included hypomagnesemia, hypertension, and a mild reversible nephropathy. A more severe nephrotoxicity was observed in 2% of the patients. Four patients responded to treatment after the addition of cyclosporine.

Cyclosporine A has also been shown to increase the response rates in refractory pediatric solid tumors (C9) as well as in hematological malignancies (L7, S22). Sonneveld *et al.* (S22) reported a 58% response rate in *MDR1*-positive myeloma patients treated with VAD plus cyclosporine. List *et al.* (L7) reported a 70% comlete response rate in AML patients. The dose-limiting toxicities associated with cyclosporine were nausea and vomiting, hypomagnesemia, and a prolongation of myelosuppression. Transient hyperbilirubinemia was observed in 62% of the treatment courses.

Cyclosporine has been shown to alter the pharmacokinetics of a number of antineoplastic agents, including VP-16 and doxorubicin (E4, L11). Lum *et al.* (L11) reported that cyclosporine increased the area under the curve (AUC) for VP-16 by decreasing both renal and nonrenal clearance of the drug. The AUC for doxorubicin was also increased in the presence of cyclosporine, again by a decrease in the clearance of the drug (E4). Such observations identify confounding variables when attempting to assess whether the observed increases in response rates are due to inhibition of the P-glycoprotein or to higher systemic levels of the cytotoxic agent.

8.5.3. *Other Agents*

While verapamil and cyclosporine A have been the major drugs studied in clinical trials to date, other agents have also been investigated. Many of these studies, however, are difficult to interpret, due either to the lack of inclusion of proper controls or to the failure to assess P-glycoprotein or *MDR1* expression.

Fojo *et al.* (F7) assessed the efficacy of the antiarrhythmic agents amiodarone and quinidine as modulators of MDR in the treatment of six patients with various refractory malignancies. They observed no complete responses and two partial responses. None of the patients developed cardiac toxicity, but chemotherapy-related toxicities, including myelosuppression, were enhanced. Quinidine was studied in a phase I study by Jones *et al.* (J1), who evaluated the addition of quinidine to epirubicin in 31 patients with advanced breast cancer. Each patient received 250–1000 mg of quinidine orally for 4 days before and 1 day after epirubicin administration. Dosages >500 mg twice daily were associated with cinchonism, nausea, and lethargy. Response rates were not reported.

Quinine, the optical isomer of quinidine, has been studied in combination with mitoxantrone and cytarabine in patients with refractory ANLL (S21). Eight of 14 evaluable patients achieved a complete response (57%), with an additional two patients achieving a partial response. P-glycoprotein expression was detected in five of 13 patients prior to treatment, and a response was reported for all. The dose-limiting toxicity was severe myelosuppression. Nonhematological toxicities associated with quinine in this study were tinnitus and vertigo in 67% of the patients and hearing loss in 40% and were considered to be acceptable.

8.5.4. *Potential Problems Related to Chemosensitization*

Because *MDR1* is expressed in normal tissues, it is conceivable that toxicity from the chemotherapeutic agent might be increased in these tissues in the presence of a chemosensitizer. Horton *et al.* (H17) studied the effect of verapamil on vincristine pharmacokinetics and toxicities in mice. They found that verapamil markedly increased the uptake and retention of vincristine in the small intestine, liver, and kidney. It is conceivable that the addition of an agent such as verapamil to a chemotherapeutic regimen might actually increase the concentration of these agents in the brain due to the expression of P-glycoprotein in the endothelial cells of capillary blood vessels and that an increased neurotoxicity of the vinca alkaloids may be manifested. It is therefore noteworthy that, in the clinical studies performed to date, no untoward neurological side effects have been reported with the vinca alkaloids and MDR chemosensitizers.

The distribution of P-glycoprotein in the liver and the kidneys suggests the possibility of altered excretion of MDR-related antineoplastic agents. As previously discussed for cyclosporine, the data suggest that modulation of P-glycoprotein function may alter the pharmacokinetic behavior of antineoplastic agents transported by this molecule. Such effects are not limited to cyclosporine A, however; verapamil has been shown to alter the pharmacokinetics of anthracyclines by increasing the AUC (K9, N6).

The vulnerability of normal tissues expressing P-glycoprotein to injury remains unknown, but this does represent a potential toxicity. Animal models such as the transgenic mouse expressing the human *MDR1* gene (M17) or the murine–human xenograft models (B12, H17) should be useful in determining the safety and efficacy of such an approach.

Hu *et al.* (H19) reported a synergistic interaction *in vitro* between verapamil and cyclosporine A in human leukemia cells displaying the MDR phenotype. This interaction was observed at clinically relevant concentrations of both drugs. Whether such synergy will be observed *in vivo* remains to be determined. To date, however, no single agent or combination has been shown to be superior in its ability to modulate resistance due to P-glycoprotein.

9. Non-P-Glycoprotein-Mediated Multidrug Resistance

It is possible that several mechanisms other than drug accumulation may play a role in MDR. These could include (1) a change in the subcellular distribution of a drug which would alter the drug concentration at the target, (2) alterations in the drug target, (3) differences in DNA repair capabilities, or (4) changes in cellular metabolic systems which would facilitate detoxification of the agent(s). Such mechanisms may be operating independently or in concert with the observed alteration in drug accumulation to contribute to the resistant phenotype.

9.1. At-MDR

Studies by Beck *et al.* (B7) and Danks *et al.* (D6) have demonstrated a non-P-glycoprotein-mediated multidrug-resistant subline of the CCRF–CEM human T-cell leukemia line; VM-5, which was selected for resistance to the epipodophyllotoxin VM-26. Although this cell line does not express the *MDR1* gene product, it displayed a cross-resistance profile which was very similar to the MDR1 phenotype, with several important exceptions. First, in contrast to the "classical" MDR phenotype, these cells remained sensitive to the vinca alkaloids, and second, there appeared to be no alteration in drug accumulation between the drug-resistant cells and their drug-sensitive counterparts. They have termed this form of resistance "At-MDR," and it appears that it is the result of an alteration in the enzyme DNA topoisomerase II. Danks *et al.* (D7) have shown that the catalytic and cleavage activities of topoisomerase II are decreased in these cells relative to the drug-sensitive parental cells. There also appears to be a decrease in both the amount and activity of the nuclear matrix-associated topoisomerase II in these cells (F2). Others have now reported cell lines which display the At-MDR phenotype (B15, D10, H6, M7, R4, S27, W3). The clinical relevance of this phenotype is currently unknown.

9.2. Other Non-P-Glycoprotein-Mediated MDR Cell Lines

The literature on non-P-glycoprotein-mediated MDR is growing steadily. In two human tumor cell lines selected for resistance to mitoxantrone, there was a

marked energy-dependent decrease in drug accumulation, yet there was no expression of the *MDR1* gene or its product (D1, T2). In contrast to the At-MDR phenotype, these cells were also resistant to the vinca alkaloids. The addition of verapamil or other MDR chemosensitizers had no effect on resistance in these cells. Several lung cancer cell lines have been described which were selected for resistance to doxorubicin and display a similar phenotype (B2, C23, M21, V8) as well as a human fibrosarcoma cell line (S20). The mechanism(s) responsible for resistance in these cell lines is as yet fully understood and may be multifactorial (S19, Z4). Somatic cell fusion studies of SW-1573 human small-cell lung cancer cell lines have demonstrated that the observed defect in drug accumulation in these cells was genetically linked to the absence of *MDR1* expression but was independent of an observed decrease in topoisomerase II expression (E1).

Several alterations have now been described in the non-P-glycoprotein cell lines. Scheper *et al.* (S6) have reported the overexpression of a 110-kDa vesicular protein in several non-P-glycoprotein MDR cell lines, but the function of this protein remains to be determined.

Cole *et al.* (C22) have described the expression of a novel member of the ATP superfamily in the H69AR human lung cancer cell line. This gene, referred to as MRP, is distantly related to the *MDR1* gene. It was found to be overexpressed by 100- to 200-fold in the resistant cell line and was decreased in a revertant line. *MRP* has also been reported to be overexpressed in other cell lines, including HT1080/DR4 and HL-60/ADR (K15, S19). The *MRP* gene codes for a message of 7.8–8.2 kb and has been localized to chromosome 16p13.1 (C22). The product of the *MRP* gene is a 190-kDa membrane-bound glycoprotein (K15). Using an RNase protection assay, Zaman *et al.* (Z1) studied *MRP* expression in a number of non-P-glycoprotein MDR human lung cancer cell lines and found that there was no detectable expression in 10 different SW-1573-derived cell lines and only modest overexpression in one GLC4-derived line. Whether or not *MRP* is playing a causative role in drug resistance awaits the results of transfection experiments, but it does not appear to be accounting for all forms of non-P-glycoprotein-mediated MDR.

Thus, while the P-glycoprotein seems to be expressed in many of the cell lines created in the laboratory, its expression is not a universal feature among MDR cell lines and suggests that mechanisms other than those associated with *MDR1* expression may be involved in maintaining the resistant phenotype.

10. Summary

Multidrug resistance represents a major obstacle in the successful therapy of neoplastic diseases. Studies have demonstrated that this form of drug resistance occurs in cultured tumor cell lines as well as in human cancers. P-glycoprotein

appears to play an important role in such cells by acting as an energy-dependent efflux pump to remove various natural-product drugs from the cell before they have a chance to exert their cytotoxic effects. Using the tools of molecular biology, studies are beginning to reveal the true incidence of multidrug resistance, as mediated by the *MDR1* gene, in the clinical setting. It has been demonstrated, at least in the laboratory, that resistance mediated by P-glycoprotein may be modulated by a wide variety of compounds, including verapamil and cyclosporine A. These are compounds which, by themselves, generally have little or no effect on the tumor cells, but when used in conjuncton with antineoplastic agents act to decrease, and in some instances eliminate, drug resistance. The mechanism(s) by which these agents act to reverse resistance is not fully understood.

Clinical trials to modulate P-glycoprotein activity are now under way to determine whether such strategies will be feasible. The detection of the P-glycoprotein in patient samples is very important in the design of these studies, as it appears that drug-resistant cells lacking P-glycoprotein will be unaffected by agents such as verapamil. Clinical studies are needed in which patients are stratified into chemotherapy protocols based on levels of *MDR1* mRNA or P-glycoprotein expression in the primary tumors.

Several research areas have been identified that are important to the transfer of the discovery of the *MDR1* gene and its protein product from the research laboratory to the clinical environment. There is an immediate need for comprehensive information on the prevalence and levels of expression of the human *MDR* genes and their protein products in human organs and tissues. Data are needed on P-glycoprotein levels in specific subpopulations (e.g., according to age, sex, race, and diet), and the study of the heterogeneity and variability of expression of P-glycoprotein in normal human tissues should be given high priority.

Since early studies have indicated some successes in identifying patients with classic multidrug resistance who might be responsive to chemosensitization, it can be anticipated that clinical research will accelerate in this area. The next wave of clinical studies will provide clinical investigators with opportunities to develop and evaluate P-glycoprotein tests and correlate test results with clinical outcomes.

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